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Fasciola hepatica infection in humans: overcoming problems for the diagnosis

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Abstract

Fascioliasis is a zoonosis actually considered as a foodborne trematode disease priority by the World Health Organization. Our study presents three cases of *F. hepatica* infection diagnosed by direct, indirect and/or imaging diagnostic techniques, showing the need of the combined use of them. In order to overcome some difficulties of the presently available methods we show for the first time the application of molecular tools to improve human fascioliasis diagnosis by employing a PCR protocol based on a repetitive element as target sequence. In conclusion, diagnosis of human fascioliasis has to be carried out by the combination of diagnostic techniques that allow the detection of infection in different disease phases, different epidemiological situations and known/new transmission patterns in the actual scenario.

Keywords

Fascioliasis, polymerase chain reaction, enzyme-linked immunosorbent assay

Introduction

Fascioliasis is an infection caused by two flukes of the genus *Fasciola*, namely *Fasciola hepatica* and *Fasciola gigantica*. *Fasciola hepatica* is present in Europe, Asia, Africa, the Americas and Oceania, whereas *F. gigantica* is restricted to Africa except the Maghreb and Asia except the high altitude areas of the Himalayas (Ashrafi *et al.* 2014). It is a common worldwide distributed disease of livestock animals as cattle and sheep, incidentally affecting humans. In the last decades, human case reports have increased in many countries and this zoonosis is actually considered as a foodborne trematode disease priority by the World Health Organization (World Health Organization, 2013). Fürst *et al.* (2012) recently estimated that more than 2.6 million people are infected by *Fasciola* sp. in the world. The major health problems are known in the Andean and Caribbean areas, northern Africa, the Near East, Southeast Asia, and Western Europe (Mas-Coma *et al.* 2015).

The definitive host is infected by ingestion of metacercariae. After ingestion, metacercariae excyst in the small intestine and migrate through the intestinal wall and Glisson's capsule to the liver. They migrate to the biliary ducts where they become sexually mature. In humans, the period from ingestion of metacercariae until sexual maturity is at least 3–4 months and life span of the adult fluke is estimated in 9 to 13.5 years. Matured liver flukes release unembrionated eggs which reach the external environment via the biliary system to the intestine. Then the eggs become embryonated and upon reaching water they release miracidia. The miracidia swim rapidly invading freshwater snails of the family Lymnaeidae as their intermediate hosts. The development in the snail includes miracidium penetration, sporocyst formation, redial generations, production and releasing of cercariae into water. Subsequently cercariae encyst as metacercariae when contacting a solid support, mostly leaves of aquatic vegetation (Mas-Coma and Bargues 2015). Infection sources include ingestion of plants and drinking of contaminated water (Mas-Coma *et al.* 2015).

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Human fascioliasis has four distinct clinical periods. The first one corresponds to the incubation phase, from the ingestion of metacercariae to the appearance of the symptoms. The second one is the invasive or acute phase, which corresponds to the fluke migration up to the bile ducts, often characterized by fever, right upper quadrant pain, unspecific gastrointestinal and respiratory signs and symptoms and urticaria. The third phase is the latent one, corresponding to the maturation of the parasites and oviposition beginning. In this phase patients can be asymptomatic or have prominent eosinophilia, gastrointestinal complaints, or one or more relapses of the acute symptoms. The last is the obstructive or chronic phase, which may develop after months to years of infection. Adult flukes in the bile ducts cause inflammation, hyperplasia of the epithelium, and thickening and dilatation of duct and gallbladder walls. During this phase biliary colic, epigastric pain, nausea, jaundice, pruritus, right upper quadrant abdominal pain and lithiasis of the bile duct or the gallbladder are frequent (Mas-Coma *et al.* 2015).

The diagnosis of human fascioliasis requires the use of direct parasitological techniques, immunological tests, and/or other imaging studies. Although detection of eggs in feces is considered to be the gold standard, eggs are undetectable during the first three phases of the disease and during the chronic phase egg detection rate is not so high because of the low egg production of the parasite, few fluke adults, intermittent egg output dynamics, and/or flukes unable to attain maturity in human subjects in nonhuman endemic areas (Mas-Coma *et al.* 1999a).

Immunological techniques can be used during all phases of the disease mainly during the acute phase in which coprological techniques will be negative. Most of these techniques detect circulating antibodies, and only few detect circulating antigens and immune complexes. Enzyme-linked immunosorbent assays (ELISA) employing purified excretory/secretory antigens and/or recombinant proteins, mainly cysteine proteinases, are the preferred assays for human serodiagnosis of *F. hepatica* (O'Neill *et al.* 1999; Carnevale *et al.* 2001; Espinoza *et al.* 2007).

Imaging techniques such as computerized tomography (CT) and ultrasonography (US) are used to confirm the diagnosis or in follow-up to evaluate therapy. In acute fascioliasis, CT findings include multiple subcapsular small nodular or branching lesions in the liver. In chronic fascioliasis, irregular thickening of the common bile duct wall, biliary dilatation and gallbladder wall thickening are usually visualized by US. Abdominal US may also show mobile vermiform structures within the gallbladder and in the bile ducts, representing flukes (Aksoy *et al.* 2005). Invasive techniques, such as endoscopic retrograde cholangiopancreatography (ERCP) may be normal in acute and latent phases or may closely mimic primary sclerosing cholangitis in the chronic phase. ERCP has also therapeutic potential in the management of fascioliasis allowing extraction of the parasites and resolution of symptoms (Ezzat *et al.* 2010).

A literature search about human fascioliasis in Argentina identified 58 reports accounting for 619 cases, since 1924 until 2009. The diagnosis was based mainly on egg detection since the beginning until now, followed by serological techniques from 2005 onwards, intradermal reaction during the 60´s, fluke surgical findings during the 70´s and 80´s, and erratic fluke observation sporadically (Mera y Sierra *et al.* 2011).

The aims of this paper are to present three cases of *F. hepatica* infection that were evaluated by different methods, including stool examination, ELISA, US and/or ERCP, and to show for the first time the application of molecular tools to improve human fascioliasis diagnosis.

Materials and Methods

Medical history

Case 1

A 52-year old female patient was admitted to a trade union clinic for routine control. She was a housewife living in a farm located in Coronel Pringles Department, San Luis province, Argentina (latitude 32°55´05″S, longitude 65°49´47″W) for the last 33 years. The water supply for consumption was boiled surface water, unfiltered. She had contact with animals including cattle, horses, poultry, dogs and cats. The patient reported picking and eating local watercress in the rural area during all year. She had not travelled outside Argentina for about 20 years.

At the time of the study the patient was symptomatic, with a three-year history of intermittent right upper quadrant pain, diarrhea, vomiting and urticaria. The clinical picture presented in episodic form. Physical examination revealed hepatomegaly. She had undergone previous abdominal US without diagnosis.

Laboratory findings revealed an elevated total white blood cell count of 19,300 leukocytes/µl (normal value 5,000-10,000 white blood cells/µl) with a marked eosinophilia of 80%. Liver function tests including serum bilirubin, alanine aminotransferase and aspartate aminotransferase were within normal limits, while alkaline phosphatase level was increased to 260 U/l (reference 40–129 U/l).

Stool examination showed the presence of *F. hepatica* eggs with a count of 100 eggs per gram of feces.

A repeated abdominal US depicted a gallbladder enlarged with a thickened wall and the presence of floating echoes. The US also showed thickening and dilatation of bile ducts, with a linear echogenic image of about 2.6 cm in length resembling a worm located in the common bile duct (Fig. 1. a, b).

Immunodiagnosis of *F. hepatica* infection by ELISA resulted positive with absorbance value of 0.480 (cut-off 0.410).

The patient was unsuccessfully treated with bithionol with a dose of 30 mg/kg daily, divided into two oral doses for 15 days. Feces remained positive for *F. hepatica* eggs after bithionol treatment and ELISA test was also positive. There-

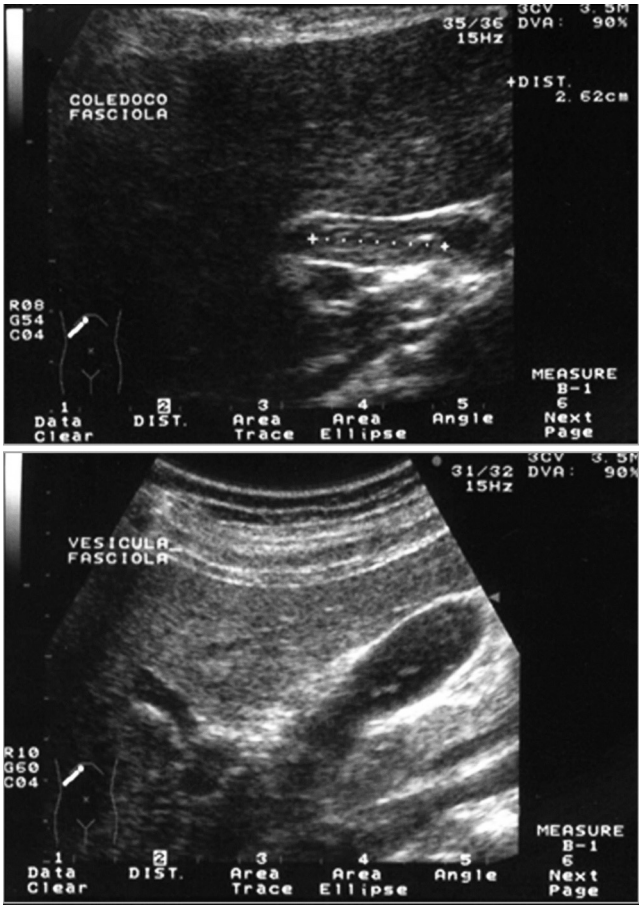

Fig. 1. a. US of the the common bile duct of patient 1 with *F. hepatica* infection depicting a linear echogenic parasite of about 26 mm. **b.** US showing floating echoes in the gallbladder of patient 1 with fascioliasis

after, the patient was treated with nitazoxanide (triclabendazole as Egaten is not registered in Argentina) at a dose of 500 mg twice a day for 7 days, but her feces remained positive for *F. hepatica* eggs. A second 7-day nitazoxanide treatment course was administered. Six weeks after treatment parasites were not seen any more with the US investigation. Fecal examination was negative for *F. hepatica* eggs after one month of the completion of treatment. IgG titers decreased but remained positive during 9 months.

Case 2

A 54-year old female patient consulted for abdominal pain. She was living in a small village from the Tinogasta Department, Catamarca province, Argentina (latitude 27°202′22″S, longitude 67°31′55″W), in contact with sheep, horses, donkeys and llamas. The water supply for consumption was unfiltered surface water. She had not traveled outside Catamarca. The patient had a five-year history of intermittent localized pain in the upper right quadrant of the abdomen, nausea and vomiting. The clinical picture of biliary colic appeared three or more times a year. Physical examination revealed hepatomegaly. The patient had been cholecystectomiced three years before.

Blood tests revealed a normal total white blood cell count of 6,700 leukocytes/µl (normal value 4,500–11,000 white blood cells/µl) with an eosinophilia of 4%. Levels of serum bilirubin, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase were normal. Stool examination was negative for ova and parasites.

An abdominal US was undergone showing a non-dilated proximal common bile duct (4 mm), with the presence of a linear echogenic image of 12 mm in length (Fig. 2), compatible with a fluke. *F. hepatica* was suspected since the presence of the parasite in animals was known in the region.

Titers of IgG determined by ELISA against *F. hepatica* were detected (absorbance value of 0.451 with cut-off 0.410).

The patient was treated with nitazoxanide at a dose of 500 mg twice a day for 7 days. Fecal examination remained negative and IgG titers decreased until negativation.

Case 3

A 22-year old female patient who consulted for presenting headache, abdominal pain, vomiting and urticaria was admitted to a public hospital in Catamarca. She lived in a rural area of Belén Department, Catamarca province, Argentina (latitude 26°35′25″S, longitude 66°55′50″W), at an altitude of about 3,600 meters above sea level (masl), where *F. hepatica* infection in animals is well-known by the inhabitants. She had undergone a cholecystectomy 7 years previously and had episodes of biliary colic attributed to residual lithiasis. Physical examination showed jaundice and right upper quadrant pain that radiated into the lower back.

Laboratory tests showed a moderate eosinophilia of 11%, as well as elevated liver function tests with a total bilirubin of 2.6 mg/dl (reference 0.00–1.00 mg/ml), alanine aminotransferase 100 U/l (reference <40 U/l) aspartate aminotransferase 64 U/l (reference <42 U/l) and alkaline phosphatase 447 U/l

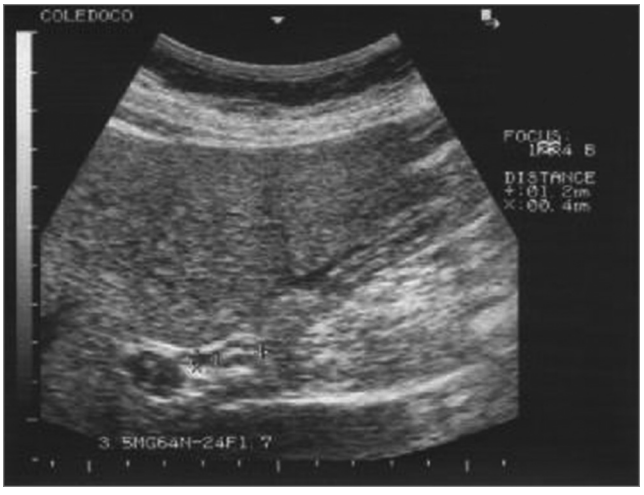

Fig. 2. US examination of patient 2 showing a linear echogenic image of 12 mm size in the proximal portion of the common bile duct

(reference 65–300 U/l). Serum electrophoretic proteinogram was normal.

Parasitological examination of feces was negative. ELISA tests for *F. hepatica* were also negative (including *in-house* and commercial devices).

Keeping in view the clinical and laboratory manifestations, an ERCP was done which showed slightly dilated extrahepatic bile duct with filiform images in the distal portion of the common bile duct. Papillotomy was performed and three living flukes, later identified as *F. hepatica*, were captured and taken out via a dormia basket.

During the following six months coproparasitological tests and serology for *F. hepatica* continued negative. Blood examinations revealed an improvement in the liver function tests and in the eosinophilia that decreased to 6%. Levels of serum bilirubin, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase became normal after three months since parasites were removed. Follow-up is being carried out by US, blood and liver function tests once a year.

Parasitological and immunological diagnosis of fascioliasis

Stool specimens were collected daily for seven consecutive days in 5% formalin saline solution. Feces were concentrated by ethyl ether centrifugation (Telemann 1908) and examined by light microscopy. If negative results were obtained, additional concentration methods of spontaneous sedimentation were employed (Dennis *et al.* 1954; Lumbreras *et al.* 1962). When *F. hepatica* eggs were detected, intensity of infection was measured as eggs per gram by the Stoll method (Stoll and Hauscheer 1926).

Serum samples were employed for immunodiagnosis of fascioliasis by ELISA using as antigen recombinant procathepsin L1 of the parasite in order to determine IgG titers (Carnevale *et al.* 2001). Plates were read on an ELISA iMark (Bio-Rad) plate reader at an absorbance of 410 nm. If negative results were obtained a commercial Fasciola Antibody Detection Test kit (SciMedx, Denville, NJ, USA) was also employed.

Molecular analysis

A pellet of 10 ml of filtered and concentrated feces was washed twice with saline solution. Thereafter DNA extraction was carried out by a standard phenol–chloroform method accordingly to our previous report (Carnevale *et al.* 2015). The DNA was finally resuspended in 20 µl of bidistilled water and kept at –20°C until use.

The presence of *F. hepatica* DNA in the stool samples was analyzed by a PCR assay using as target a repetitive element of *F. hepatica* (GenBank accession number U11819.1) and the primer set FhrepF (5′-ATTCACCCATTTCTGTTAGTCC-3′) and FhrepR (5′-ACTAGGCTTAAACGGCGTCC-3′) previously described (Kramer and Schnieder 1998) based on nucleotides 6–27 and 103–122 of the sequence.

The amplifications were carried out in a MyCycler thermocycler (Biorad, USA) in 50 µl volumes under the following final conditions: 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08% (v/v) Nonidet P40, 100 µM (each) dNTP, 2.5 mM $MgCl_2$, 0.5 µM of each primer, 1.0 U of Taq DNA polymerase (Fermentas International Inc.), and 0.048% BSA. PCR templates corresponded to 2 µl of purified DNA diluted 1:200 in bidistilled water. The PCR protocol included an initial step of 3 min at 98°C, followed by 30 cycles of denaturation for 60 sec at 95°C, annealing for 60 sec at 56°C, and extension for 90 sec at 72°C, with a final step of 3 min at 72°C. A second round of amplification was added employing 5 µl of the products of the first round, under the same cycling conditions.

The negative control corresponded to DNA from feces of an asymptomatic human living in a non-endemic area, with coprological and serological negative assays for *F. hepatica*. Positive control was DNA from *F. hepatica* adult specimen extracted with NucleoSpin DNA Tissue Kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). For the blank reaction mixture DNA template was replaced by distilled water. Twenty microliters of amplicons from the second round were analyzed by electrophoresis in a 2.5% agarose gels, stained by ethidium bromide and visualized under UV illumination.

The research protocol was approved by the Ethical Committee for Research, Hospital Francisco J. Muñiz, protocol N° 267. Informed consent in writing was obtained from each patient.

Results

The operculated eggs found in the stool samples from case 1 (Fig. 3) were identified as *F. hepatica* eggs on the basis of their ovoid shape and mean size (140 × 85 µm).

In case 3, three flat organisms, leaf-shaped and brownish, with measures of 1.9–2.1 × 1.0–1.2 cm were extracted. They presented a cone-like anterior extension of the body, marked shoulders of the cephalic cone, a spiny tegument, and two small suckers. They were identified as *F. hepatica* (Fig. 4).

From our three cases only one proved positive to coproparasitological analyses, while the other two cases were negative during all the follow-up.

Immunodiagnosis of *F. hepatica* using recombinant procathepsin L1 as antigen was able to detect IgG levels in cases 1 and 2. In case 1 IgG titers were still determined after coprological negativation, showing positive results for more than half a year. In case 2, with negative coprological results, anti-*F. hepatica* IgG titers decrease rapidly after treatment. ELISA was negative before and after fluke removal in patient 3 using the *in-house* test with recombinant cystein protease antigen or the commercial kit.

Molecular diagnostics allowed the detection of parasite DNA in stool samples (Fig. 5).

Feces from patient 1 containing *F. hepatica* eggs resulted positive by the double PCR assay, showing an amplification

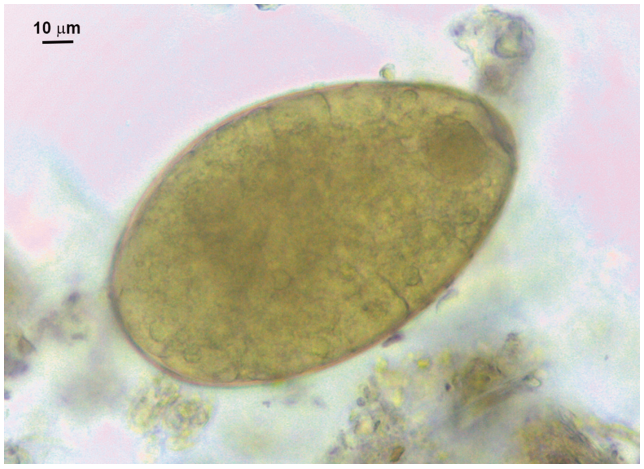

Fig. 3. Operculated egg of *F. hepatica* (140 × 85 µm) found in stool samples of patient 1 by microscopy, at 400x magnification

fragment of the expected size (about 117 base pairs). No amplification was detected with DNA from stool samples of patient 1 after the last nitazoxanide treatment with negative coproparasitological analysis, although ELISA remained positive.

Fig. 4. One of the three *F. hepatica* flukes recovered by endoscopic sphincterotomy from patient 3

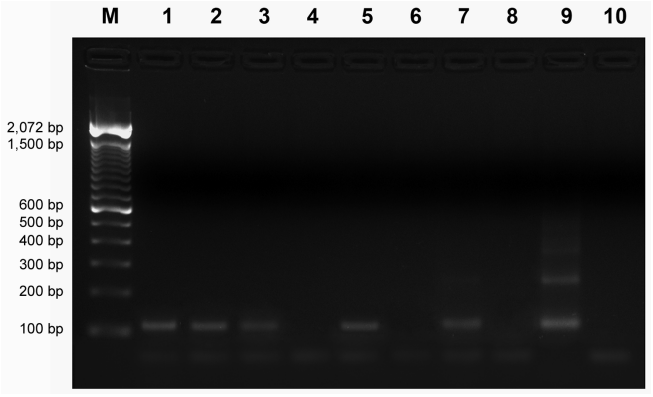

Fig. 5. Electrophoresis of amplification products after the double PCR of the repetitive element of *F. hepatica* in 2.5% agarose gel stained by ethidium bromide. Lane M, 100 bp ladder; lanes 1 to 4, patient 1 at initial diagnosis, after bithionol, after first round of nitazoxanide and after second round of nitazoxanide, respectively; lane 5 and 6, patient 2 before and after treatment with nitazoxanide; lanes 7 and 8, patient 3 before and after endoscopic sphincterotomy; lane 9, positive control (2 ng of *F. hepatica* DNA); lane 10, reaction mixture

When samples from patient 2 were analyzed, the PCR was positive in the fecal sample at the time of the US detection of the parasite, with a serological positive diagnosis and a negative stool analysis. After nitazoxanide treatment, the amplification products were not detected.

Samples from patient 3 gave amplification products before the endoscopic sphincterotomy. Stool samples were collected one month after the parasite removal procedure, and they became negative by the molecular technique.

Discussion

Fascioliasis in humans has been epidemiological classified under different categories (Mas-Coma *et al.* 1999b) corresponding to autochthonous, isolated, nonconstant cases; imported cases; hypoendemic; mesoendemic; hyperendemic; epidemics in nonhuman endemic but animal endemic areas; and epidemics in human endemic areas. Our cases 1 and 2 lived in rural human endemic areas previously described (Malandrini *et al.* 2009; Carnevale *et al.* 2013). Case 3 lived in a small rural settlement in the dry highlands of Catamarca, in a depression located between 3,200 and 5,500 masl, which epidemiological characteristics for fascioliasis have not been previously studied.

A classification of transmission patterns has also been proposed, distinguishing a very high altitude pattern in Andean countries, a Caribbean insular pattern, a pattern related to Afro-Mediterranean lowlands, a pattern related to Caspian surrounding areas and a pattern related to lowland areas in Southeast Asia (Mas-Coma 2005). In our cases 1 and 2 transmission could not be explain by the actually known epidemiological patterns of fascioliasis, as although belonging to an Andean country, the snail vectors identified until now in the studied

areas (Carnevale *et al.* 2013; Bargues *et al.* 2016) were not in concordance with the very high altitude pattern.

These different epidemiological and transmission patterns of human fascioliasis endemic areas add difficulties to the diagnosis (Mas-Coma *et al.* 2014). These problems are also pronounced by the different phases of the disease. In this sense, a recent review (Velusamy *et al.* 2004) advised for the combined use of different techniques in order to cover more needs and situations in the actual global scenario.

Our study presented three cases of *F. hepatica* infection diagnosed by direct, indirect and/or imaging diagnostic techniques, showing the need of the combined use of them. In order to overcome some difficulties of the presently available methods, we applied molecular tools to improve individual diagnosis.

Repetitive DNA sequences are a feature of most eukaryotic genomes and often represent non-coding regions (Kramer and Schnieder 1998). The *Fasciola* specific sequence described by Kaplan *et al.* (1995) is a highly repetitive element of the *Fasciola* genome without similarities to known sequences available at the GenBank database. In the present report we adapted a PCR protocol that had been previously described to amplify this repetitive DNA sequence in infected snail intermediate hosts (Velusamy *et al.* 2004). This PCR is a confirmation of *Fasciola* infection but it does not distinguish the parasite at species level as it is a genus specific assay (Kramer and Schnieder 1998). Since there is no report of *F. gigantica* in the Americas therefore, it is possible to assume that this is *F. hepatica* infection in these patients.

Our three cases show the advantages of the combined used of different approaches for the diagnosis of human fascioliasis.

Patient 1, who lived in a mesoendemic area, had a coprological analysis that revealed the presence of *F. hepatica* eggs. Diagnosis of human fascioliasis was also supported by ELISA, US and PCR. In concordance with these results and the clinical picture, case 1 seemed to be in the chronic phase of the disease. After successful treatment, stool analysis and PCR became negative but the ELISA was positive for a period longer than six month. As the immunoassay is based on the determination of circulating antibodies against the parasite, it seems to be not adequate for monitoring after treatment due to the fact that the return to negative values occurs later that the infection is resolved.

Case 2, who lived in a hyperendemic area, was diagnosed by the immunological assay but the direct analysis of feces was negative while the molecular technique resulted positive. These results were accompanied by the finding of a compatible image in the US. The ELISA test in samples from experimentally infected sheep using recombinant pro-cathepsin L1 of *F. hepatica* has allowed the detection of specific IgG levels since three weeks post-infection (Carnevale *et al.* 2001), showing the advantages of this method for the early detection of infection compared with stool analysis. This situation could be one of the explanations for this case, representing a latent phase of the disease. Another situation could be the presence of only one parasite observed by US, where the antibody detection is a very good alternative for a very low parasite burden. In both cases, the finding of eggs in feces would be not achieved for a previous phase of the disease when the parasite is immature or due to a reduced shedding. Eggs are often not found in stools in human fascioliasis (Zumaquero-Ríos *et al.* 2013; Mas-Coma *et al.* 2014). This problem could be overcome by the employment of the PCR assay, showing the presence of parasite DNA in stools that could be the result of fluke cells or undetectable eggs. After treatment, PCR became negative but the ELISA remained positive for a longer period until negative results.

Case 3, a young woman who lived in a high altitude area in which animal fascioliasis is recognized, was diagnosed by extraction of three living flukes using the ERCP. Her coprological and serological analyses were negative, while the PCR resulted positive in stool samples collected before the endoscopic treatment. As the patient had been previously cholecystectomiced and continued with episodes of biliary colic, presenting also jaundice and right upper quadrant pain, it is presumably that corresponded to a stage of high chronic obstructive fascioliasis, in which lithiasis of the bile duct or the gallbladder is frequent. In Argentina, the long delay in confirmation of *F. hepatica* infection has been reported (Mera y Sierra *et al.* 2011), nearly three and a half years in average, suggesting either infected subjects not looking for professional diagnosis and/or misdiagnosis of patients because of confusion with other diseases. Under this assumption, considering the 7 years elapsed since the patient undergone the cholecystectomy, and taking in account an estimated life span of the adult fluke between 9 and 13.5 years, determination of antibodies against *F. hepatica* could result negative due to a drastic reduction of the immunological response intensity. Although *Fasciola* egg dynamics in humans is unknown, intermittent and very low egg shedding in old infections may not be ruled out (Mas-Coma *et al.* 2014) and this could be the reason for the negative direct stool analysis in this patient. The positive result of the PCR assay could be interpreted as detection of parasite DNA in feces from adult or egg cells.

To our knowledge, this is the first report employing a molecular approach in the diagnosis of human fascioliasis, as all previous works were intended to animal hosts (Martínez-Pérez *et al.* 2012; Robles-Pérez *et al.* 2013; Carnevale *et al.* 2015). A last point that should be discuss is that this test could be compared with the use of coproantigen assays, which are able to detect infections with very few or no eggs in stools (Valero *et al.* 2012; Zumaquero-Ríos *et al.* 2013). The main inconvenience of coproantigen tests is that samples should be refrigerated or frozen because the coproantigen degrades at ambient temperature or common fixatives, carrying transportation problems or impeding to diagnose of other parasites. Although a new fecal preservative, CoproGuard™, which contains biocidal substances, proteins, and surfactants has been developed for preservation of *Fasciola* coproantigens and has been evaluated in human samples (Ubeira *et al.* 2009), its use is not widespread at present.

Our report shows the employment of formalin fixed stool samples, corresponding to the material commonly used in the parasitological examination, allowing simple conditions for transport and storage. Moreover, the assay can be carried out without the use of any commercial or *in-house* made kit, with the only requirements of equipment and reagents for the PCR technology.

The use of PCR in the diagnosis of human fascioliasis has a high value in detecting low and early infections, allows distinguishing between current and past infections and can help in the prognosis of the patients.

In conclusion, diagnosis of human fascioliasis at the individual level has to be carried out by the combination of diagnostic techniques that allow the detection of infection in different phases, several epidemiological situations and known/new transmission patterns.

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